Activation of IL-6/ERK/c-MYC signaling confers resistance to NVP-BKM120, a pan-PI3K inhibitor, in head and neck squamous cell carcinoma

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Activation of IL-6/ERK/c-MYC signaling confers resistance to NVP-BKM120, a pan-PI3K inhibitor, in head and neck squamous cell carcinoma

Directed by Professor Byoung Chul Cho

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirement for the degree of Master of Medical Science

Bomi Kim

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This certifies that the Master's Thesis

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2014 년 12 월

김보미 올림.

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ABSTRACT

Activation of IL-6/ERK/c-MYC signaling confers resistance to NVP-BKM120, a pan-PI3K inhibitor, in head and neck squamous cell carcinoma

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Although, the phosphatidylinositol 3-kinase (PI3K) signaling axis is the most frequently deregulated pathways in head and neck squamous cell carcinoma (HNSCC), therapeutic strategies targeting this pathway have been underwhelming to date. The purpose of this study is to elucidate potential mechanisms of resistance to PI3K inhibitor in HNSCC. The inhibitory effect of NVP-BKM120, a pan PI3K inhibitor, on cellular growth in 10 human HNSCC cell lines was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony formation. Functional and mechanistic studies were analyzed by means of ELISA, RT-PCR, and western blot in CAL27 and SCC15 cells among HNSCC cell lines. Treatment of NVP-BKM120 showed the limited anti-proliferative effect

with IC_{50}s in 0.5 to 1.5 micromole ranges against 10 HNSCC cell lines. In both CAL27 and SCC15 cells, the activation of PI3K downstream signaling pathways including phosphorylated-AKT and S6K was initially blocked, but the phosphorylation of AKT and S6K was restored after 24h of NVP-BKM120 treatment. Moreover, NVP-BKM120 significantly induced c-MYC expression, ERK activation, and IL-6 secretion. The knockdown of c-MYC with siRNA transfection in two HNSCC cell lines led to the increased sensitivity to NVP-BKM120. Furthermore, IL-6 receptor neutralizing antibody completely abolished NVP-BKM120-induced c-MYC expression by down-regulating ERK activation, whereas inhibition of c-MYC and ERK failed to block the increased levels of IL-6 secretion. Collectively, these results suggest that IL-6/ERK/c-MYC axis contributing to intrinsic and adaptive resistance to NVP-BKM120 may ultimately limit its efficiency of NVP-BKM120 in HNSCC cell lines. Our preclinical study provides a rationale for combination therapy of IL-6/ERK/c-MYC inhibition and NVP-BKM120.

Key words: Head and neck squamous cell carcinoma (HNSCC), PI3K signaling pathway, NVP-BKM120, drug resistance, c-MYC, IL-6, ERK

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I. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) arises in the oral cavity, oropharynx, larynx or hypopharynx, and is the sixth leading cancer by incidence worldwide.[1] It is likely that approximately 600,000 cases will arise this year worldwide, and that only 40–50% of patients with HNSCC will survive for 5 years.[2] While the mortality of HNSCC patients has decreased, patients frequently present with incurable disease, evidence that additional novel therapies are currently required.[3] In HNSCC, *EGFR* and *PIK3CA* are frequently dysregulated by genetic abnormalities.[3]

Phosphoinositide 3-kinases (PI3Ks) belong to a conserved family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides. The most well-characterized product of this reaction is phosphatidylinositol-3,4,5-trisphosphate or PIP_3, a critical second messenger that recruits AKT for activation of growth, proliferation and survival signaling.[4] Abnormal activity of PI3K signaling is one of the most frequent alterations in human cancer. This pathway is triggered by several different mechanisms in cancers, including somatic mutation and amplification of genes encoding key components. In HNSCC, numerous genetic mutations of PI3K pathway have been reported, such as *PIK3CA* mutations, PTEN somatic mutations, *PTEN* loss of heterozygosity (LOH) and loss of PTEN protein expression.[5] In addition, it was reported that PI3K signal pathway may serve integral functions for noncancerous cells in the tumor microenvironment.[6]

Consequently, significant efforts have been made to efficiently inhibit the PI3K pathway cancer treatment.[6,7] However, emerging clinical data show limited single-agent activity of PI3K inhibitors at tolerated doses.[8,9] Because of the complexity, crosstalk and feedback in the PI3K–AKT–mTOR signaling network, the inhibitory effect of PI3K pathway is limited.[10] The purposes of this study to evaluate of the anti-tumor responses to NVP-BKM120 in HNSCC, investigate the mechanisms of resistance of NVP-BKM120 and verify the relative potency of therapeutic combination to overcome the resistance to NVP-BKM120.

In this research, we have revealed the resistance mechanism of NVP-BKM120 resistance in HNSCC. Our data suggest that pan-PI3K inhibitor, NVP-BKM120, leads to the autocrine production of IL-6 and activated ERK mediated c-MYC overexpression. Our preclinical study provides a rationale for combination therapy with IL-6/ERK/c-MYC inhibition and NVP-BKM120.

II. MATERIALS AND METHODS

1. Reagents and antibodies

NVP-BKM120 (Buparlisib) was kindly provided by Norvatis. (Basel, Switzerland) Interleukin-6 receptor (IL-6R) neutralizing antibody and mouse IgG negative control were obtained from R&D Systems (Minneapolis, Minnesota, USA). All stealth siRNAs including negative low GC siRNA and serum free Opti-MEM(not added phenol red) were purchased from Invitrogen (Carlsbad, California, USA). β-actin antibody, protease inhibitor cocktail and phosphatase inhibitor cocktails for immunoblotting were purchased from Sigma-aldrich. (St. Louis, Missouri, USA) Other primary antibodies and protein lysis buffer for immunoblotting were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

2. Cell culture

CAL27 cells were cultured at 37℃ in 5% CO_2 air in Dulbeccos' Modfied Eagle's Media (DMEM; HyClone, Logan, Utah, USA) supplemented 10% heat inactivated FBS (HyClone), 100 units/mL penicillin (HyClone). SCC15 cells were maintained in same condition except Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DME/F’12) added 400ng/mL hydrocortisone (Sigma Aldrich).

3. MTT assay

To measurement cell viability, cells were plated at 0.3×10^4/well in 96-well plate. Next day, CAL 27 and SCC 15 cells were incubated 72 hours in cell cultured condition after treating BKM120. After drug exposure, 0.5mg/ml of MTT was added to the medium in the well. After incubation for 4 hours at 37℃ formazan crystals in visible cells were solubilized with 100μL/well dimethyl sulfoxide (DMSO, Duchefa, Zaandam, Netherlands). The optical density of the MTT formazan product was read at 570nm on an ELISA reader. All experiments were conducted in triplicate. MTT was purchased from Duchefa.

4. Measurement of IL-6 production

Supernatants from cultured cells were collected and centrifuged for 15 minutes at 1500rpm, 4℃ to remove dead cells and cellular debris. Following centrifugation, the supernatants were stored at -80℃. ELISA for IL-6 in supernatants was conducted in triplicate using human IL-6 ELISA kits (R&D systems) according to the manufacturer's protocols. The optical density of the IL-6 production was read at 450nm on an ELISA reader.

5. Western blot analysis

Total cellular proteins were extracted in lysis buffer added with protease inhibitor cocktail, phosphatase inhibitor cocktails and 1mM phenylmethylsulfonyl fluoride (PMSF) at 4℃. After incubation for 30 min on ice, insoluble debris was removed by centrifugation for 30 min at 4℃. Proteins were spectrophotometrically quantified with the BCA Protein Assay (Thermo Fisher Scientific, Fremont, California, USA) at 562nm. Proteins immobilized on

nitrocellulose membranes (Merck Millipore, Billerica, Massachusetts, USA) were blotted overnight at 4℃ with primary antibody. NC membranes were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). β-actin was used for loading control.

6. RNA extraction, quantitative real-time PCR and RT-PCR

Cells were washed in phosphate-buffered saline (PBS) at 4℃ three times. RNA was isolated using TRIzol reagent (Ambion, Carlsbad, California, USA), according to manual. 3μg of total RNA was reverse-transcribed with RT-premix kit. (Bioneer, Daejeon, Korea) RT PCR was performed with PCR premix kit (Bioneer). All steps were conducted according to the manufacturer's instructions. Real-time RT–PCR was performed in a 7500 Real-time PCR system (Applied Biosystems, California, USA) with SYBR Green master mix (Applied Biosystems). All reactions were done in a 20μL reaction volume in triplicate. The primers used for RT-PCR and real time PCR are as follows: *GAPDH* (forward) 5'-TTCGACAGTCAGCCGCATCTTCTT-3′ and (reverse) 5'-GCCCAATACGACCAAATCCGTTGA-3′. *IL-6* (forward) 5'-CTGCCAGTGCCTCTTTGCTG-3', and (reverse) 5'-CTTCTCCACAAGCGCCTTCG-3'**.** *c-MYC* (forward) 5'-CTTCTCTCCGTCCTCGGATTCT-3′ and (reverse) 5′-GAAGGTGATCC AGACTCTGACCTT-3′.

7. siRNA transfection

siRNA transfection was conducted with Lipofectamine RNAiMAX (Invitrogen) reagents according to manufacturer's instruction. Briefly, the cells were transfected with 20nmol/L of siRNA for 6 hours with Lipofectamine RNAiMAX reagent and replaced with fresh growth medium. The following day, cells were treated with BKM120 or DMSO for MTT assay, colony formation or immunoblot analysis.

8. Colony formation assay

After transfection, cells were seeded at 0.5×10^4/well in 6-well plate. Next day, DMSO or BKM120 1μM was treated. Colonies were washed once with cold PBS on ice. Then, colonies were fixed with 4% para-formaldehyde 30 minutes at room temperature and stained with 0.2% crystal violet (Sigma Aldrich) 6–10 days after drug treatment. Culture media was freshly changed every 72 hour.

9. Cytokine and phospho –RTK array analysis

The phospho-RTK array was performed with 300μg of total protein. 1mL of cultured media was used for cytokine array. All analyses were conducted according to manufacturer's introductions. Membranes were detected by same method of western blot analysis. The relative expression level of cytokine or phospho-RTK was determined by comparing the intensity of each spot with the negative and positive controls. These array kits were from R&D systems.

10. Statistical analysis

All results were analyzed using Student t test and $P < 0.05$ was considered statistically significant.

III. RESULTS

1. NVP-BKM120 modestly inhibits the growth of human HNSCC cell lines

To evaluate the activity of NVP-BKM120, a pan PI3K inhibitor, for multiple human HNSCC cell lines, the cells were treated with NVP-BKM120 for 72 h and then cell viability was measured by MTT assay. As shown in Figure 1A, HNSCC cell lines exhibited half maximal inhibitory concentration (IC_{50}) of NVP-BKM120 ranging from 0.5 μM to 1.5. When used 1 μM IC_{50} values as a cutoff to arbitrarily divide NVP-BKM120-sensitive and -resistant cell lines, only two cell lines (CAL27, CAL33) fell into the BKM120-sensitive cell lines. Moreover, there was no correlation between response to NVP-BKM120 and genetic alterations such as *PI3KCA* mutation. Among human HNSCC cell lines, we investigated the inhibitory effect of NVP-BKM120 on activation of PI3K pathway in CAL 27 cells harboring *NRAS* mutation and SCC15 cells harboring *KRAS* amplification. Western blot analysis revealed that NVP-BKM120 treatment effectively reduced activity of PI3K pathway, including phosphorylated-AKT, S6K, and 4EBP1 up to 24hr. However, there was restoration of PI3K activity after 24hr of NVP-BKM120 treatment (Figure 1B). These results suggest that there may be adaptive resistance mechanism to NVP-BKM120, leading to reactivation of PI3K pathway.

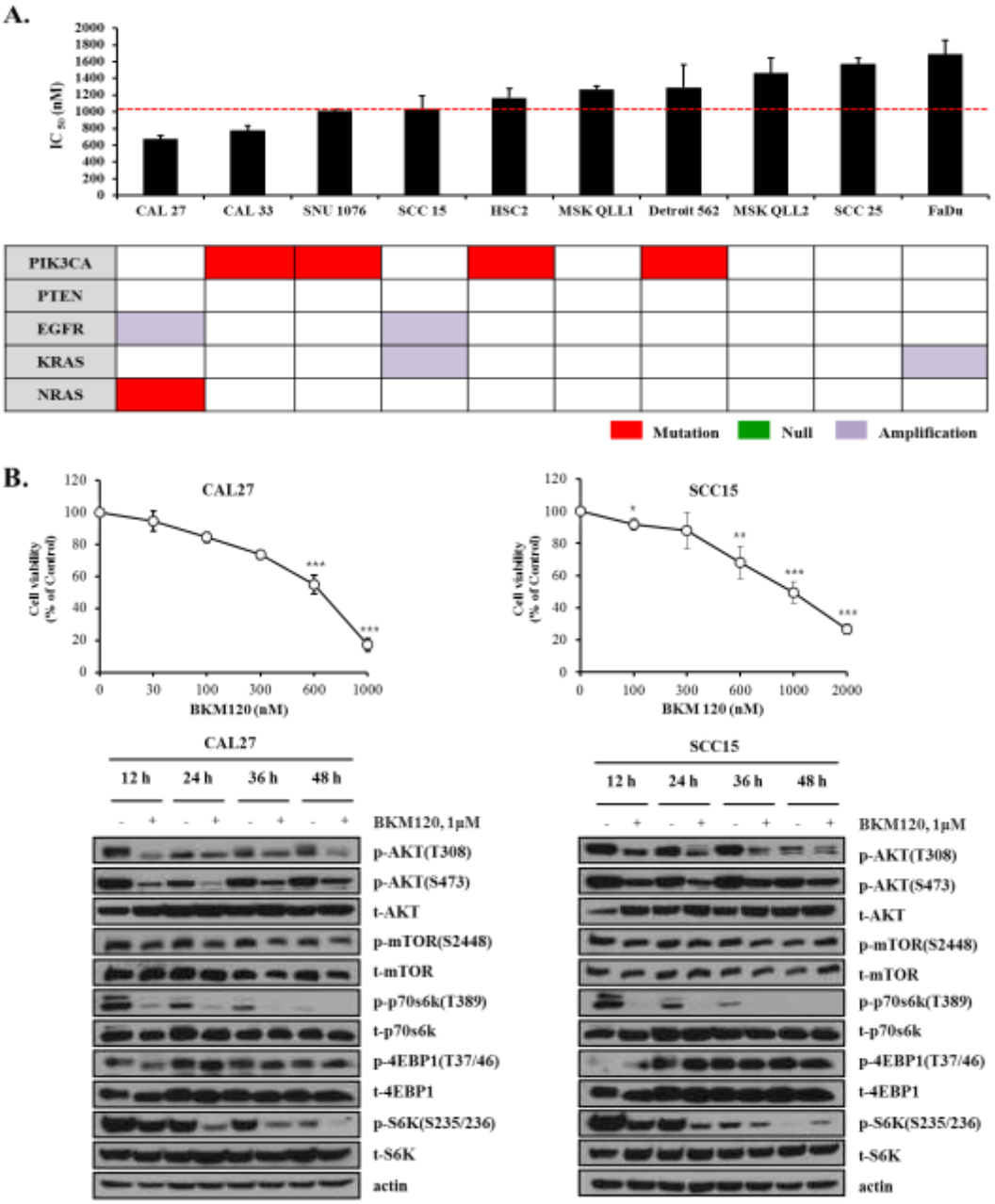

A.
IC50 (nM)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CAL 27
CAL 33
SNU 1076
SCC 15
HSC2
MSK QLL1
Detroit 562
MSK QLL2
SCC 25
FaDu
PIK3CA
PTEN
EGFR
KRAS
NRAS
Mutation
Null
Amplification
B.
CAL27
Cell viability (% of Control)
BKM120 (nM)
0
30
100
300
600
1000
SCC15
BKM 120 (nM)
2000
12 h
24 h
36 h
48 h
BKM120, 1μM
p-AKT(T308)
p-AKT(S473)
t-AKT
p-mTOR(S2448)
t-mTOR
p-p70s6k(T389)
t-p70s6k
p-4EBP1(T37/46)
t-4EBP1
p-S6K(S235/236)
t-S6K
actin

Figure 1. NVP-BKM120 modestly inhibits the growth of human HNSCC cell lines. (A) Average BKM120 IC_{50} values for the indicated 10 HNSCC cell lines. Cells were treated with increasing does of BKM120 for 72 hours and the viability was determined using MTT assay. Lower table presents the genetic mutation characters of each HNSCC cell lines. (B) Measurement of BKM120 induced cell viability inhibition by MTT assay. (upper panel) The results are the average of three independent experiments (each n=3) Asterisks indicate significant difference as follows; *$p < 0.05$, compared with control; **$p < 0.01$, compared with control; ***$p < 0.001$, compared with control. Western blotting analysis of 40μg protein lysates probed with antibodies against PI3K downstream signals ─ AKT, mTOR, p70S6K, S6K, 4EBP1 ─ in two HNSCC cells. (lower panel)

2. NVP-BKM120 induces c-MYC expression by controlling transcriptional regulation

On the basis of potential oncogenic function of c-MYC in human cancer and frequent overexpression of c-MYC in HNSCC[11], we determined whether treatment of NVP-BKM120 could affect the expression of c-MYC in two cell lines (CAL27 and SCC15) using real-time PCR and western blot analysis. Treatment of 1μM induced c-MYC mRNA expression in both these cell lines in time dependent manner (Figure 2A). Upon NVP-BKM120 treatment, the level of c-MYC protein was also markedly elevated in the two cells (Figure 2B). Since JQ1, a BET bromodomain inhibitor, is known as potential inhibitor of c-MYC[12, 13], we hypothesized that combination with JQ1 could enhance the sensitivity of cells to NVP-BKM120 treatment by regulating c-MYC expression. To this end, we assessed combination effects of JQ1 with NVP-BKM120 on cell growth. As shown in Figure 3, when the cells were treated with JQ1 alone, both CAL27 and SCC15 cells were resistant against JQ1 (we used 5 μM IC50 as a cutoff). In contrast, combination of NVP-BKM120 at various does (30-100nM) and 1μM JQ1 exhibited a strong synergistic effect on cell growth inhibition. To confirm these results, the cells were transfected with control or c-MYC siRNA, treat them with NVP-BKM120, and measured cell growth. Consistent with above results, cell proliferation was effectively inhibited by a combination of siMYC and NVP-BKM120 compared with either treatment alone, indicating that c-MYC may be a key oncogenic factor contributing to the limited efficacy of NVP-BKM120 in human HNSCC.

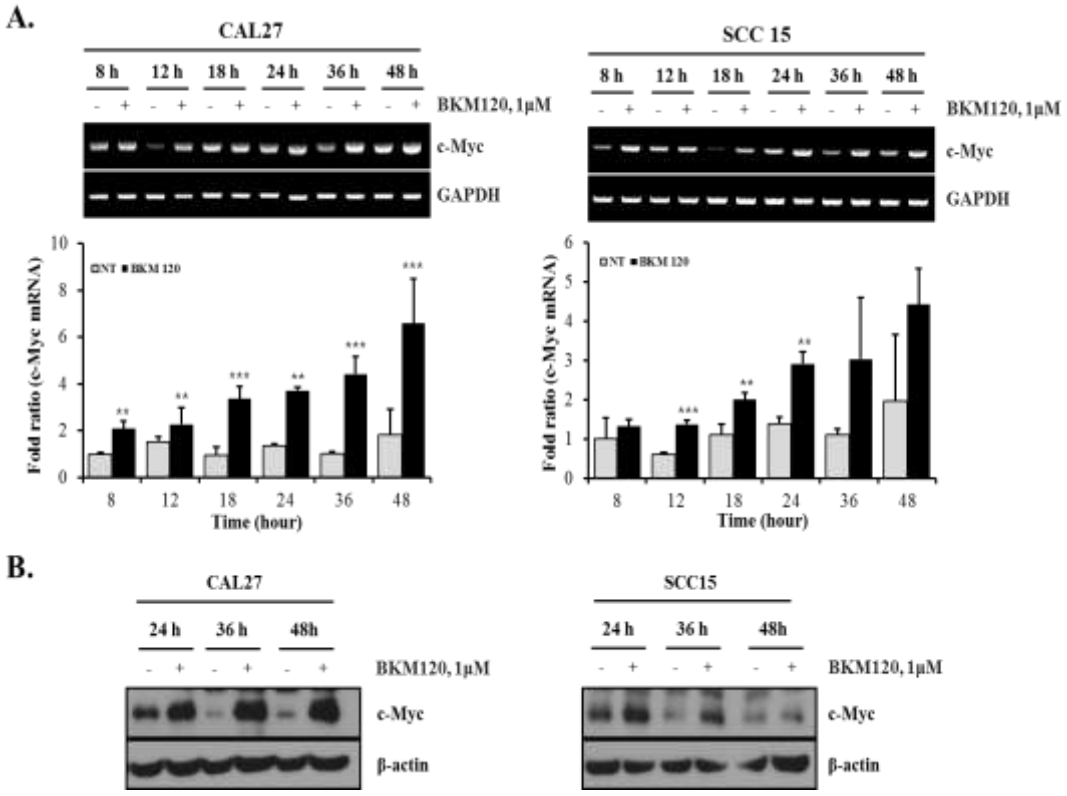


Figure 2. NVP-BKM120 dramatically increases c-MYC expression by controlling transcriptional regulation. (A and B) Time point expression of c-MYC mRNA and protein in DMSO or BKM120 1μM exposed HNSCC cell lines. The mRNA level of IL-6 was detected by RT-PCR (upper panel of A) and qPCR (lower panel of A). GAPDH was used as loading control. (B) HNSCC cells were treated with DMSO or BKM120 1μM and cells were lysed at indicated time points (hours). Total cell lysates (25μg) were then measured by Western blot for analyzing of expression about c-MYC. Actin was used for loading control.

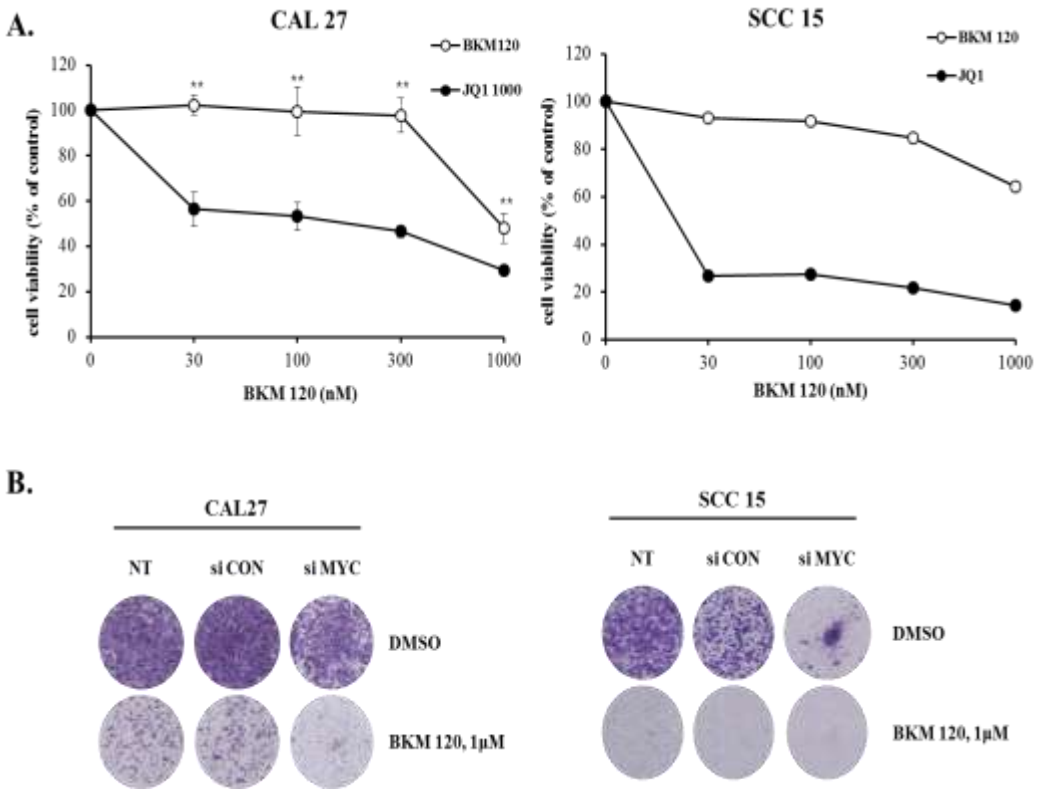
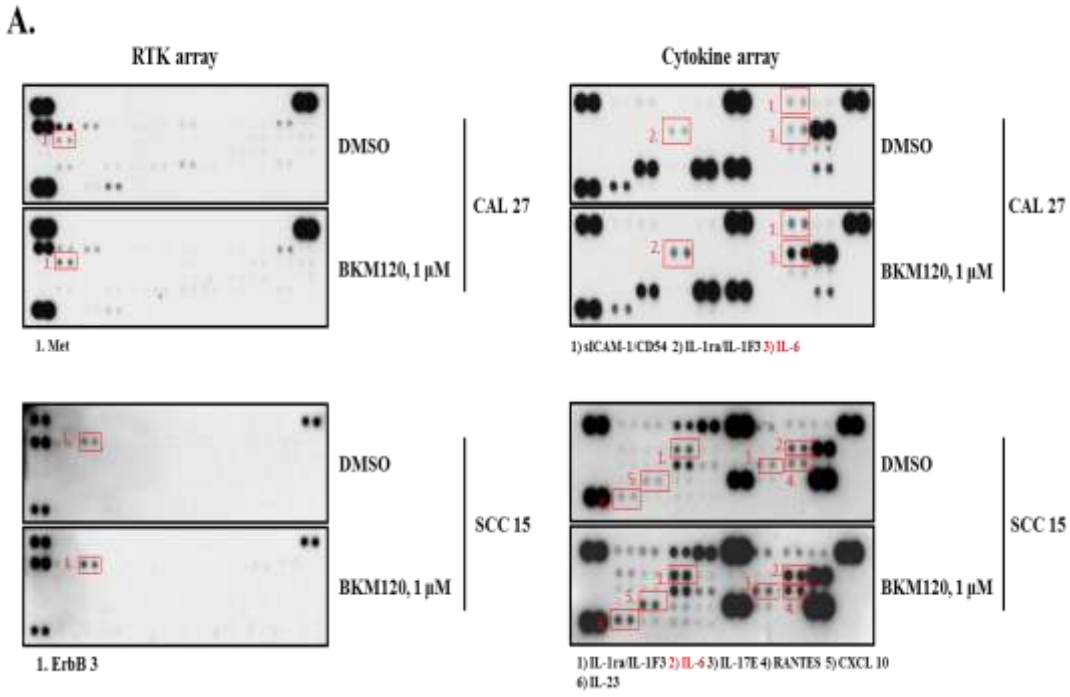
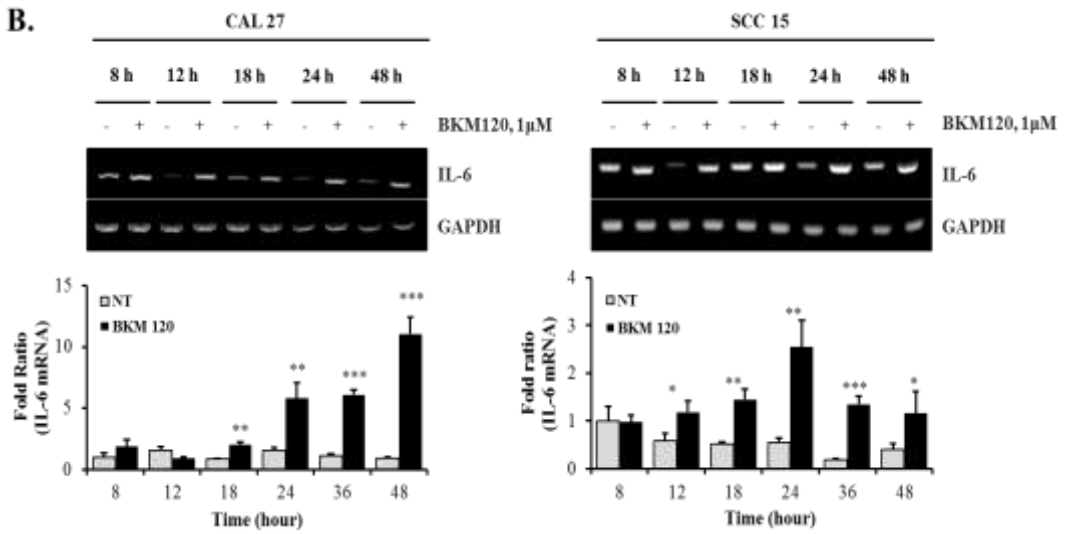


Figure 3. c-MYC may be a key oncogenic factor contributing the limited efficacy of NVP-BKM120. (A) The cells were seeded into 96-well plate (3 x 10^3 cells per well) and treated with NVP-BKM120 or combination of NVP-BKM120 and JQ1 at the indicated concentrations. At 72 hours, MTT assay was performed to determine the effect of co-treatment of BKM120 and JQ1. (B) Colony formation assay of HNSCC cell lines. Cells were seeded into 6-well plate (5 x 10^3 cells per well). Next day, they were treated with NVP-BKM120 incubation with DMSO, control siRNA or c-MYC siRNA for 10 days. After finishing the incubation, each well was stained with 0.23% crystal violet, and photographed. Experiments were performed in triplicate.

3. NVP-BKM120-induced c-MYC expression is mediated by IL-6 autocrine signaling

To identify the signal transduction pathway(s) controlling the NVP-BKM120-induced c-MYC expression, we performed phospho-RTK and cytokine array in cells treated with NVP-BKM120 for 24 h. As shown in RTK array results, there was no significant activation of RTKs upon NVP-BKM120 in both two cell lines. In contrast, cytokine array results demonstrated that NVP-BKM120 induced the secretion of several cytokines and chemokines in these cells. Among all cytokines/chemokines, only IL-6 was significantly increased in both cell lines (Figure 4A). In line with these results, qPCR and western blot also showed that NVP-BKM120 significantly increased the levels of IL-6 mRNA and protein in a time-dependent manner (Figure 4B and C). Reportedly, IL-6 production is implicated in resistance to anticancer drugs by regulating several oncogenic factors such as survivin and bcl-xL.[14,15] Thus, we asked if autocrine IL-6 secretion mediates c-MYC overexpression. To address this question, we examined the NVP-BKM120-induced c-MYC overexpression upon inhibition of IL-6 signaling. As shown Figure 4D, the blockade of IL-6 signaling using IL-6 receptor neutralizing antibody completely interrupted NVP-BKM120-induced c-MYC expression, whereas both JQ1 treatment and silencing of c-MYC failed to prevent NVP-BKM120-mediated IL-6 secretion. These results suggested that IL-6 may mediate NVP-BKM120-induced c-MYC expression in human HNSCC.


A.
RTK array
Cytokine array
DMSO
BKM120, 1 μM
CAL 27
SCC 15
1. Met
1) sICAM-1/CD54 2) IL-1ra/IL-1F3 3) IL-6
1. ErbB 3
1) IL-1ra/IL-1F3 2) IL-6 3) IL-17E 4) RANTES 5) CXCL 10 6) IL-23


B.
CAL 27
SCC 15
8 h
12 h
18 h
24 h
48 h
BKM120, 1μM
IL-6
GAPDH
NT
BKM 120
Fold Ratio (IL-6 mRNA)
Fold ratio (IL-6 mRNA)
Time (hour)

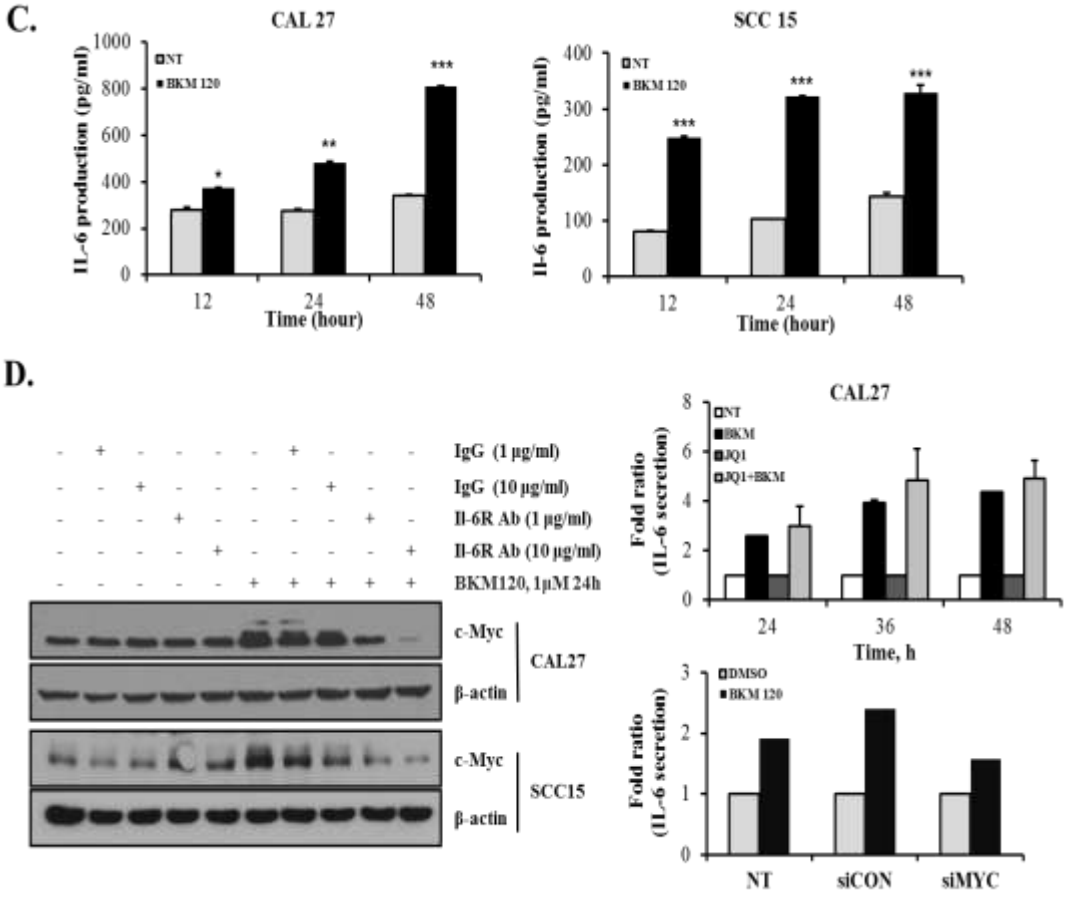

C.
CAL 27
SCC 15
IL-6 production (pg/ml)
Il-6 production (pg/ml)
NT
BKM 120
Time (hour)
12
24
48
D.
IgG (1 μg/ml)
IgG (10 μg/ml)
Il-6R Ab (1 μg/ml)
Il-6R Ab (10 μg/ml)
BKM120, 1μM 24h
c-Myc
β-actin
CAL27
SCC15
CAL27
NT
BKM
JQ1
JQ1+BKM
Fold ratio (IL-6 secretion)
Time, h
DMSO
BKM 120
NT
siCON
siMYC

Figure 4. NVP-BKM120-induced c-MYC expression is mediated by IL-6 autocrine signaling. (A) Levels of phospho-RTK and cytokines secreted by CAL27 and SCC15 cells, as determined using phospho-RTK and cytokine array. 300μg of protein was used for phospho-RTK array and cytokine array was performed with 1ml of cultured media. Cell lysate protein or cultured media was incubated with phosphor-RTK or cytokine array membranes. Detection of membranes was accomplished using the anti-phospho-tyrosine-HRP or streptavidin-HRP antibody and enhanced chemi reagent mix. (B and C) Time point expression of IL-6 mRNA and protein in HNSCC cell lines was shown. (B) The mRNA level of IL-6 was detected by RT-PCR (upper panel) and qPCR (lower panel). GAPDH was used as loading control. (C) Secreted IL-6 production in the two cell lines was analyzed by ELISA. Asterisks indicate significant difference as follows; *$p < 0.05$, compared with control; **$p < 0.01$, compared with control; ***$p < 0.001$, compared with control. (D) (left panel) CAL27 and SCC15 cells were incubated for 1 hour in the presence of IL-6 receptor neutralizing antibody. Afterwards, DMSO or BKM120 1μM added in both HNSCC cells and they were cultured for 24 hours. Cell lysates were examined for c-MYC expression by Western blot analysis. (right panel) Measurement of IL-6 secretion level in CAL27 cells not silenced and silenced c-MYC by ELISA assay. c-MYC was knocked down by JQ1 1μM treatment or siRNA transfection. Before treatment of BKM120 1μM, JQ1 1μM or DMSO exposed CAL27 cells were incubated for 1 hour. Negative control siRNA or c-MYC siRNA transfected cells were treated BKM120 for 24 hours. After finishing each incubation, 1ml of cultured was collected on ice and used for IL-6 ELISA assay.

4. The increased secretion of IL-6 mediates c-MYC expression via ERK activation

Since IL-6 mediates its function via JAK/STAT pathway, Ras/ERK pathway, and PI3K/AKT pathway[16], specific pharmacologic inhibitors for these pathways were tested to verify direct mediator involved in IL-6-modulated c-MYC expression. As shown in Figure. 5A, NVP-BKM120 mediated c-MYC expression was markedly attenuated by U1026, a MEK inhibitor, but not by P6, a pan-Jak inhibitor. In parallel, after treatment with NVP-BKM120, ERK was strongly phosphorylated in time-dependent manner, whereas activation of STAT3 was not observed (Figure 5B). Moreover, IL-6 receptor neutralizing antibody led to a significant reduction of ERK phosphorylation (Figure 5C). Collectively, these results suggest that IL-6/ERK signaling axis might participate in regulation of NVP-BKM120-induced c-MYC expression.

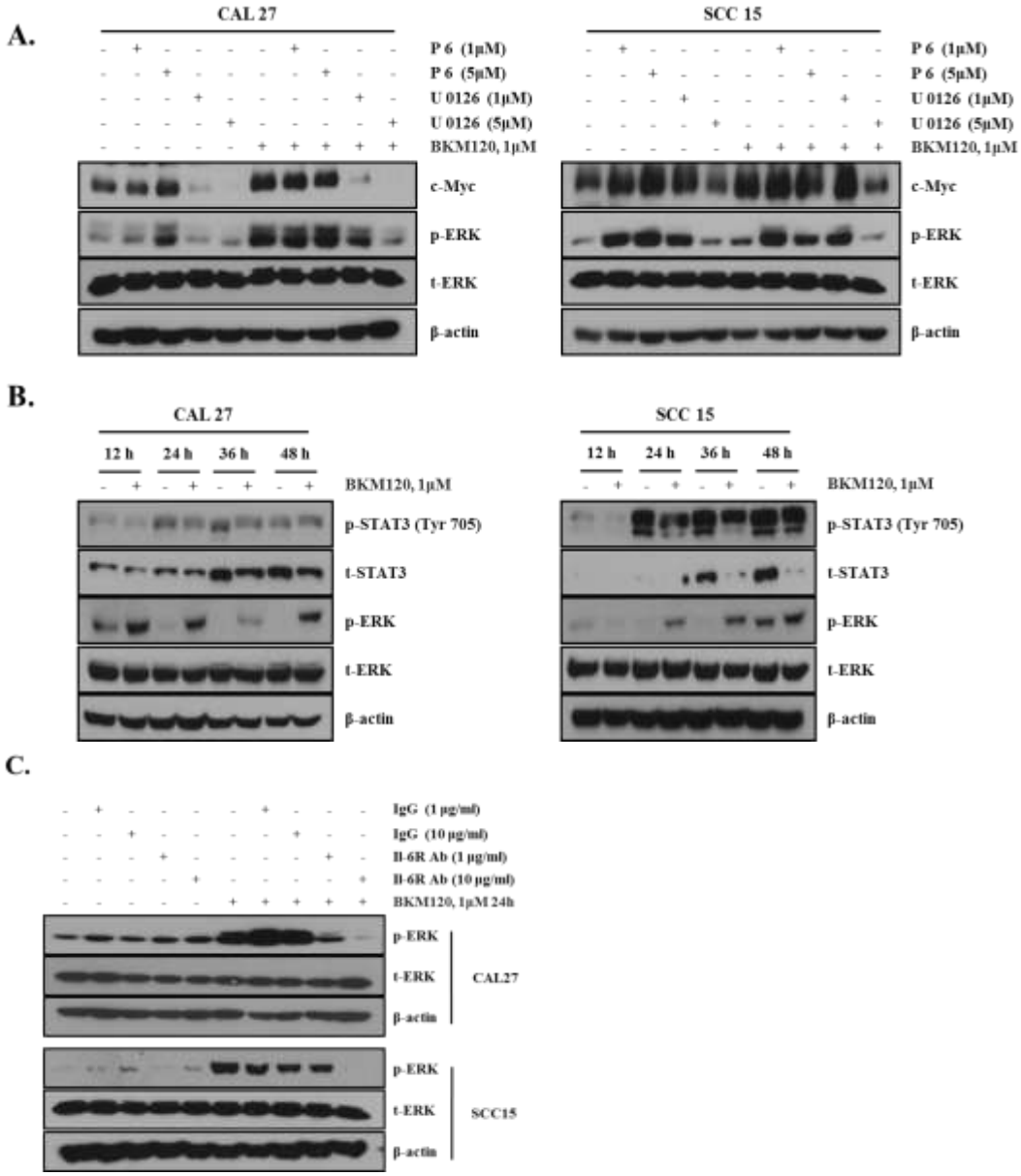

A.
CAL 27
SCC 15
P 6 (1μM)
P 6 (5μM)
U 0126 (1μM)
U 0126 (5μM)
BKM120, 1μM
c-Myc
p-ERK
t-ERK
β-actin
B.
CAL 27
SCC 15
12 h
24 h
36 h
48 h
BKM120, 1μM
p-STAT3 (Tyr 705)
t-STAT3
p-ERK
t-ERK
β-actin
C.
IgG (1 μg/ml)
IgG (10 μg/ml)
Il-6R Ab (1 μg/ml)
Il-6R Ab (10 μg/ml)
BKM120, 1μM 24h
p-ERK
t-ERK
β-actin
CAL27
SCC15

Figure 5. The increased secretion of IL-6 mediates c-MYC expression via RAS/ERK activation. (A) CAL27 and SCC15 cells were treated with P6 at indicated concentrations (1 or 5 μM) and U0126 (1 or 5 μM) or BKM120 1μM for 24 hours. The cell lysates were examined for expression of c-MYC and pERK by Western blot analysis. (B) HNSCC cells were treated with BKM120 1μM and cells were lysed at indicated time points (hours). Total cell lysates (40μg) were then examined by Western blot analysis for STAT3 and ERK. Actin was used as loading control. (C) CAL27 and SCC15 cells were incubated for 1 hour in the presence of IL-6 receptor neutralizing antibody. Afterwards, DMSO or BKM120 1μM added in both HNSCC cells and they were cultured for 24 hours. Cell lysates were examined for pERK and ERK expression by Western blot analysis.

IV. DISSCUSION

PI3K pathway regulates cell proliferation, differentiation, cellular metabolism, and cytoskeletal reorganization.[3,13] Therefore, various PI3K inhibitors have been clinically developed.[6,7,17] The clinical efficacy of PI3K inhibitors has been reported to be modest, most probably due to adaptive resistance mechanisms.[7,8] For instance, mTORC1 inhibition leads to activation of PI3K signaling through a feedback loop, and this has been proposed as a possible reason for the restraint efficacy of rapamycin in epithelial cancers other unknown reasons of diminishing anti-tumor effect of PI3K drugs must be revealed to overcome resistance to anticancer therapies.[18] Futhermore, JAK2/STAT5-evoked positive feedback loop that dampens the efficacy of PI3K/mTOR inhibition. PI3K/mTOR inhibition increased IRS1-dependent activation of JAK2/STAT5 and secretion of IL-8 in several cell lines and primary breast tumors.[19] Therefore, other causes of diminishing anti-tumor effect of PI3K inhibitors must be revealed to overcome PI3K drug resistance.

In this study, we report that targeting PI3K pathway drug induced overexpression of c-MYC. There have been several studies regarding the role of c-MYC in the resistance of PI3K inhibitors.[20,21,22] The anti-proliferation effect of BKM120 (Figure 1A) and rapid restoration of PI3K downstream signals (Figure 1B) in two HNSCC cells supports the findings that cancer cells previous exhibit rapid adaptation to PI3K inhibition through multiple feedback mechanisms. Moreover, BKM120 increased mRNA and protein expression level of c-MYC (Figure 2A-B). MYC is a pleiotropic transcription factor that has been linked to a diverse range of cellular functions. Not surprisingly, aberrant MYC

signaling has been observed in human cancers and MYC has been reported to promote cell transformation and carcinogenesis.[23,24] To confirm whether c-MYC is main factor of diminishing anti-tumor effect of BKM120 in HNSCC, MTT assay using BKM120 and BET bromodomain inhibitor, JQ1, that can disrupt expression of c-MYC and colony formation with c-MYC transient knock down transfection showed synergistic anti-proliferation effect (Figure 3A-B). Our results suggest c-MYC is pivotal role in the development of resistance to NVP-BKM120.

It has been frequently reported that dysregulation of IL-6 is related to the cause of many types of cancer and triggers the chemoresistance of multiple myeloma, renal cell carcinoma, cholangiocarcinoma, prostate cancer, and breast cancer cells.[14,25,26] However, the mechanisms of drug resistance by IL-6 secretion in HNSCC has not yet been fully clarified. At first, we investigated the difference of phosphor-RTK or cytokine level in BKM120-treated HNSCC cells. In RTK array (Figure 4A), IL-6 secretion was higher in BKM120 treated HNSCC cells than DMSO-treated HNSCC cells. Overexpression of IL-6 both in mRNA and protein level was confirmed by RT-PCR, real time PCR and IL-6 ELISA (Figure 4B-C). These results reinforce that secretion of IL-6 by autocrine manner is the main event in the development of diverse drug resistance to NVP-BKM120.

The precise mechanism of regulation of c-MYC expression by IL-6 has been poorly understood. At first, we investigate whether the expression of c-MYC is affected by IL-6 signal inhibition. As shown in Figure 4D, IL-6 blockade by IL-6R antibody in two HNSCC

cell lines presented dramatically decreased protein expression of c-MYC. We further tested if c-MYC could regulate the IL-6 secretion level. Neither pharmacologic inhibition of c-MYC by JQ1 nor siMYC treatment did not restrain the secretion of BKM120-induced IL-6 secretion. To summarize these results, IL-6 is an upstream regulator of the expression of c-MYC.

The main downstream signals of IL-6 are as follows; JAK/STATs, Ras/MEK/ERK and PI3K/AKT/mTOR.[16] Some researches addressed that activated STAT3 triggered by IL-6 induced expression of c-MYC.[27,28] To test the previous results, we checked the phospholylation status of Y705-STAT3 in BKM120-treated HNSCC cells using western blot. As shown in Figure 5B, the activity of STAT3 suppressed in BKM120 presence, but phospholylation of ERK was increased. ERK1/2 are serine-threonine kinases and their activities are positively regulated by MEK1 and MEK2.[29] ERKs can directly phosphorylate many transcription factors including Ets-1, c-Jun and c-MYC.[29,30] Therefore, we hypothesized ERK could regulate the expression of c-MYC in BKM120-treated HNSCC. To verify our hypothesis, we performed further immunoblotting for p-ERK and c-MYC following BKM120 treatment with or without pan-JAK inhibitor (P6) and MEK inhibitor (U0126). In both cell lines, U0126 dramatically decreased the expression of c-MYC, whereas P6 did not (Figure 5A). Taken together, ERK is the upstream regulator of c-MYC.

Ras is a main signaling molecule triggered by interleukin-6. [31,32] However, there have been very few published reports about its specific action mechanism. We found that IL-6

autocrine loop of HNSCC cells activated ERK and BKM120 induced ERK was almost completely blocked by IL-6 receptor neutralizing antibody. (Figure 5C) Our results suggest that IL-6 dependent Ras/MEK/ERK signaling may decrease the sensitivity to NVP-BKM120 through overexpressing c-MYC.

Taken together, our study provides a mechanism that HNSCC cell lines develops pan PI3K drug resistance. Identification of the molecular mechanism that regulates the protein or mRNA levels of c-MYC provides a new strategy to suppress MYC-mediated drug resistance.

V. CONCLUSION

Collectively, these results suggest that activation of IL6/ERK/c-MYC axis may contribute to adaptive resistance to NVP-BKM120, limiting the efficacy of NVP-BKM120. The preclinical studies provide a rationale for combination therapy of IL-6/ERK/c-MYC inhibition and NVP-BKM120.

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ABSTRACT (in Korean)

두경부암에서 IL-6/ERK/c-MYC 신호 전달 경로의 활성화가 pan-PI3K 경로 억제제인 BKM120 내성 형성에 기여하는 기작에 관한 연구

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phosphatidylinositol 3-kinase (PI3K) 신호 전달계는 두경부암에서 가장 빈번하게 비정상적으로 조절되는 신호 전달 경로이지만, 지금까지 이 경로를 차단하는 치료법 수립이 미미한 현황이다. 그러므로, 이 연구의 목적은 두경부암에서 PI3K 저해제에 보이는 내성 기전을 밝히는 데에 있다. 10 종류의 인간 두경부암 세포주에서 Pan PI3K 저해제인 NVP-BKM120 이 보이는 세포 성장 억제 효과를 MTT assay 와 colony formation 으로 측정하였다. 두경부암 세포주 중 CAL27 과 SCC15 를 선택하여, ELISA, RT-PCR, Western blot 을 통해 약물의 기능과 기전을 연구하였다. 10 종류의 인간 두경부암 세포주들에 NVP-BKM120 를 처리했을 때, 그 IC_{50} 값이 0.5~1.5 μM 로 제한적인 종양

억제 효과를 보였다. CAL27 과 SCC15 세포주에서, PI3K 하위 신호인 phosphorylated-AKT 와 phosphorylated-S6Kinase 가 처음에는 억제가 되었지만, 이렇게 감소되었던 PI3K 신호 전달 경로가 NVP-BKM120 처리를 한지 24 시간이 지난 후에는 점진적으로 다시 증가함을 확인하였다. 더불어 NVP-BKM120 은 c-MYC 의 발현과 ERK 의 활성 그리고 IL-6 의 분비를 증가시켰다. 두 가지의 두경부암 세포주에서 c-MYC siRNA 를 이용하여 c-MYC 유전자를 제거하였을 때, NVP-BKM120 에 대한 항암 효과가 증가한 것을 확인하였다. 나아가 IL-6 수용체 항체를 이용하여 저해하였을 때, ERK 활성화가 감소됨과 동시에 NVP-BKM120 에 의해 증가한 c-MYC 의 발현이 떨어졌지만, c-MYC 과 ERK 의 저해는 IL-6 의 분비량을 감소시키지 못했다. 이러한 결과들을 종합해보면, 두경부암에서 IL-6-ERK-c-MYC 신호 전달 경로의 활성화는 NVP-BKM120 에 대한 약제 효과를 떨어뜨린다고 할 수 있다. 따라서, 이번 연구 결과는 IL-6-ERK-c-MYC 억제제와 NVP-BKM120 병용 치료에 대한 근거를 제시할 것으로 생각된다.

핵심되는 말 : 두경부암, PI3K 신호 전달 경로, NVP-BKM120, 항암제 내성, c-MYC, IL-6, ERK